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Comparative cytogenetic analysis among filistatid spiders (Araneomorphae: Haplogynae)

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Abstract. The family Filistatidae is considered sister to Synspermiata or sister to Hypochilidae. Cytogenetic knowledge of this family could be useful for understanding the mechanism of chromosome evolution that has occurred within the group. In this work, two filistatid species belonging to distinct subfamilies, *Kukulcania hibernalis* (Hentz, 1842) (Filistatinae) and *Misionella mendensis* (Mello-Leitão, 1920) (Prithinae), were investigated using standard and differential chromosome staining. Analysis of mitotic and meiotic cells revealed the diploid $2n♂ = 25$ for *K. hibernalis* and $2n♂ = 21$ for *M. mendensis*. Both species exhibited a sex chromosome system of the X_1X_2Y type and metacentric/submetacentric chromosomes. In prophase I cells, the sex chromosomes were in a trivalent configuration with all elements associated without chiasma through their terminal regions. Both species revealed six nucleolar organizer regions on the terminal region of three autosomal pairs. In *K. hibernalis*, constitutive heterochromatin was located mainly in the terminal regions of autosomes and sex chromosomes while in *M. mendensis*, the heterochromatin occurred in the pericentromeric region of all chromosomes. Despite the scarcity of cytogenetic information for Filistatidae, the available results show the occurrence of high variability in the diploid number but with the maintenance of the X_1X_2Y sex chromosome system. Additionally, the karyotype differentiation in the species of this family seems to have involved not only the number of autosomes but also specific chromosomal sites, such as the constitutive heterochromatic regions.

Keywords: constitutive heterochromatin, karyotype, meiosis, nucleolar organizer region, sexual trivalent

The spider family Filistatidae is composed of 147 species and 19 genera, having a worldwide distribution, with the greatest diversity in tropical and subtropical biogeographic regions (Gray 1995; Ramírez & Grismado 1997; World Spider Catalog 2016). The phylogenetic position of Filistatidae is in dispute. While some studies on spinneret morphology (Platnick et al. 1991) and respiratory system morphology (Ramírez 2000) point towards a sister relationship between Filistatidae and the ecribellate haplogynes (=Synspermiata as proposed in Michalik & Ramírez 2014), forming the Haplogynae clade, others, focusing on phylogenomics (Bond et al. 2014; Garrison et al. 2016), placed Filistatidae (*Kukulcania* Lehtinen, 1967) as sister to basal araneomorph Hypochilidae (*Hypochilus* Marx, 1888).

There are few studies regarding the phylogenetic relationships between the genera of Filistatidae. However, Gray (1995) and Ramírez & Grismado (1997) subdivided the family into Filistatinae and Prithinae. The subfamily Filistatinae comprises three genera, *Filistata* Latreille, 1810, *Kukulcania*, and *Sahastata* Benoit, 1968, whereas Prithinae includes *Afrofilistata* Benoit, 1968, *Andoharano* Lehtinen, 1967, *Filistatinella* Gertsch & Ivie, 1936, *Filistatoides* F. O. Pickard-Cambridge, 1869, *Lihuelistata* Ramírez & Grismado, 1997, *Misionella* Ramírez & Grismado, 1997, *Pikelinia* Mello-Leitão, 1946, *Pritha* Lehtinen, 1967, *Wandella* Gray, 1994, and *Yardiella* Gray, 1994, totaling ten genera (Ramírez & Grismado 1997). This phylogenetic hypothesis did not include the six other filistatid genera, *Antilloides* Brescovit, Sánchez-Ruiz & Alayón, 2016, *Microfilistata* Zonstein, 1990, *Mystes* Bristowe, 1938, *Pholcoides* Roewer, 1960, *Tricalamus* Wang, 1987 and *Zaitunia* Lehtinen, 1967 (World Spider Catalog 2016).

Only three species of Filistatidae have been cytogenetically investigated, *Filistata insidiatrix* (Forsskål, 1775) from Greece, with $2n♂ = 33$, X_1X_2Y, *Kukulcania hibernalis* (Hentz, 1842) from Argentina, and *Kukulcania* aff. *hibernalis*, which showed karyotypes with $2n♂ = 24$, X_1X_20 and $2n♂ = 25$, X_1X_2Y, respectively. In these three species, the chromosomes exhibited metacentric and submetacentric morphology (Rodríguez Gil et al. 2002; Král et al. 2006; Kořínková & Král 2013). In the present work, two species of Filistatidae belonging to different subfamilies, *Kukulcania hibernalis* (Filistatinae) and *Misionella mendensis* (Mello-Leitão 1920) (Prithinae), were analyzed using standard and differential chromosome staining techniques. This is the first cytogenetic study of *M. mendensis* and the identification of specific chromosome regions in filistatid spiders. Taking into account that Filistatidae is likely relatively basal within araneomorph spiders, the cytogenetic knowledge of this family is useful for understanding the mechanisms of chromosome evolution that occurred within the group.

METHODS

The cytogenetic data of the Brazilian Filistatidae examined in this work corresponded to a sample of eight male and four female individuals of *K. hibernalis* from Barra do Jacaré (23°06′54″S, 50°10′51″W), state of Paraná, and three male specimens and four embryos (three males and one female) of *M. mendensis* collected in the municipalities of Rio Claro (22°24′39″S, 47°33′39″W) and São Paulo (23°32′52″S,

* *In memoriam*

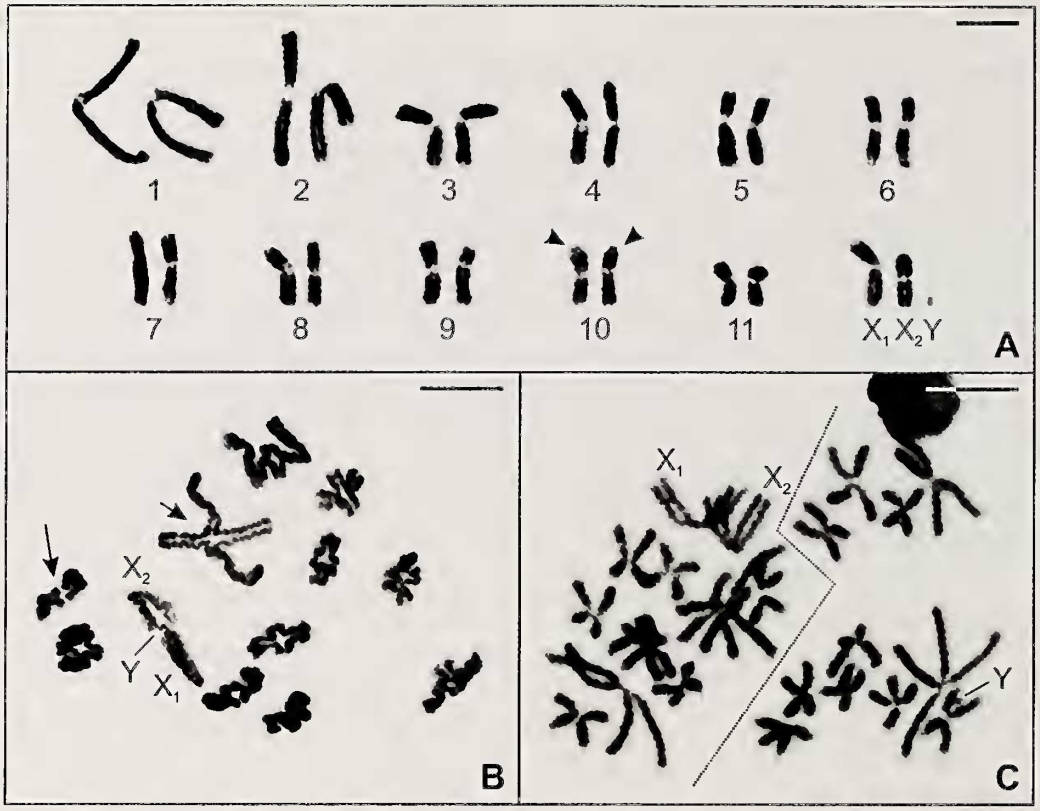

Figure 1.—Testicular cells of *Kukulcania hibernalis* stained with Giemsa. A. Karyotype, $2n♂ = 25$, X_1X_2Y, with metacentric chromosomes. Note the heteromorphism of the chromosomes of pair 9. Arrowheads indicate secondary constrictions on pair 10. B. Diplotene, exhibiting bivalents with interstitial (small arrow) or terminal (large arrow) chiasmata. C. Metaphase II, showing n = 13, X_1X_2 (left pole) and n = 12, Y (right pole). Scale = 10 μm.

46°38′9″W), state of São Paulo. The voucher specimens were deposited in the collection of the Laboratório Especial de Coleções Zoológicas, Instituto Butantan (IBSP, curator A. D. Brescovit), São Paulo, Brazil. The chromosomal preparations were obtained from embryos and gonads of adult individuals, following the methodology described by Araujo et al. (2005). All the cytological preparations were standard stained with 3% Giemsa solution (3% of commercial Giemsa and 3% of phosphate buffer pH 6.8 in distilled water). For identification of the nucleolar organizer regions (NORs) and location of constitutive heterochromatin regions, chromosome preparations were submitted to silver impregnation (Howell & Black 1980) and C-banding (Sumner 1972), respectively. To obtain a better resolution of the C-banding pattern, chromosomes were stained with 4-6′-diamidino-2-phenylindole (DAPI). All cells were photographed using an Olympus BX51 light microscope coupled to an Olympus DP71 digital camera with DP Controller software. The chromosomes were measured using LEVAN, a plugin for Image J software, developed by Sakamoto & Zacaro (2009), and morphologically classified following Levan et al. (1964).

RESULTS

Kukulcania hibernalis.—Mitotic metaphase cells of *K. hibernalis* revealed the diploid number 2n = 25 for males and 2n = 26 for females. The comparative study of both mitotic cells of the males and females and metaphase II cells of the males, showed the presence of a sex chromosome system of the $X_1X_2Y/X_1X_1X_2X_2$ type for this species (Fig. 1). The male karyotype was composed of chromosomes of large (pairs 1 and 2), medium (pairs 3 to 10) and small (pair 11) sizes. The X_1 and X_2 sex chromosomes were medium-sized whereas the Y chromosome was extremely small, being identified as the smallest element of the karyotype. All chromosomes showed a metacentric morphology, with the exception of one element of pair 9, which was classified as submetacentric (Fig. 1A). This morphological heteromorphism of pair 9 was verified in three male specimens, in which a detailed karyotype analysis was accomplished. A secondary constriction was easily visualized in the short arm terminal region of pair 10.

Early prophase I nuclei exhibited three (two large and one small) positive heteropycnotic blocks of sexual chromatin. Diplotene cells exhibited 11 autosomal bivalents with one terminal or interstitial chiasma (Fig. 1B), except the large bivalent that occasionally presented two chiasmata, one terminal and one interstitial. The sex chromosomes were easily identified since they formed a trivalent configuration, with all elements associated through their terminal regions. In this association, both arms of the X_1 and X_2 chromosomes were in an achiasmate pairing with both arms of the Y chromosome, which always assumed a central position in this trivalent configuration.

Silver-impregnated spermatogonial metaphase cells revealed six NORs located on the short arm terminal region of pairs 1, 5 and 10 (Fig. 2A, B). However, not all NORs appeared in the

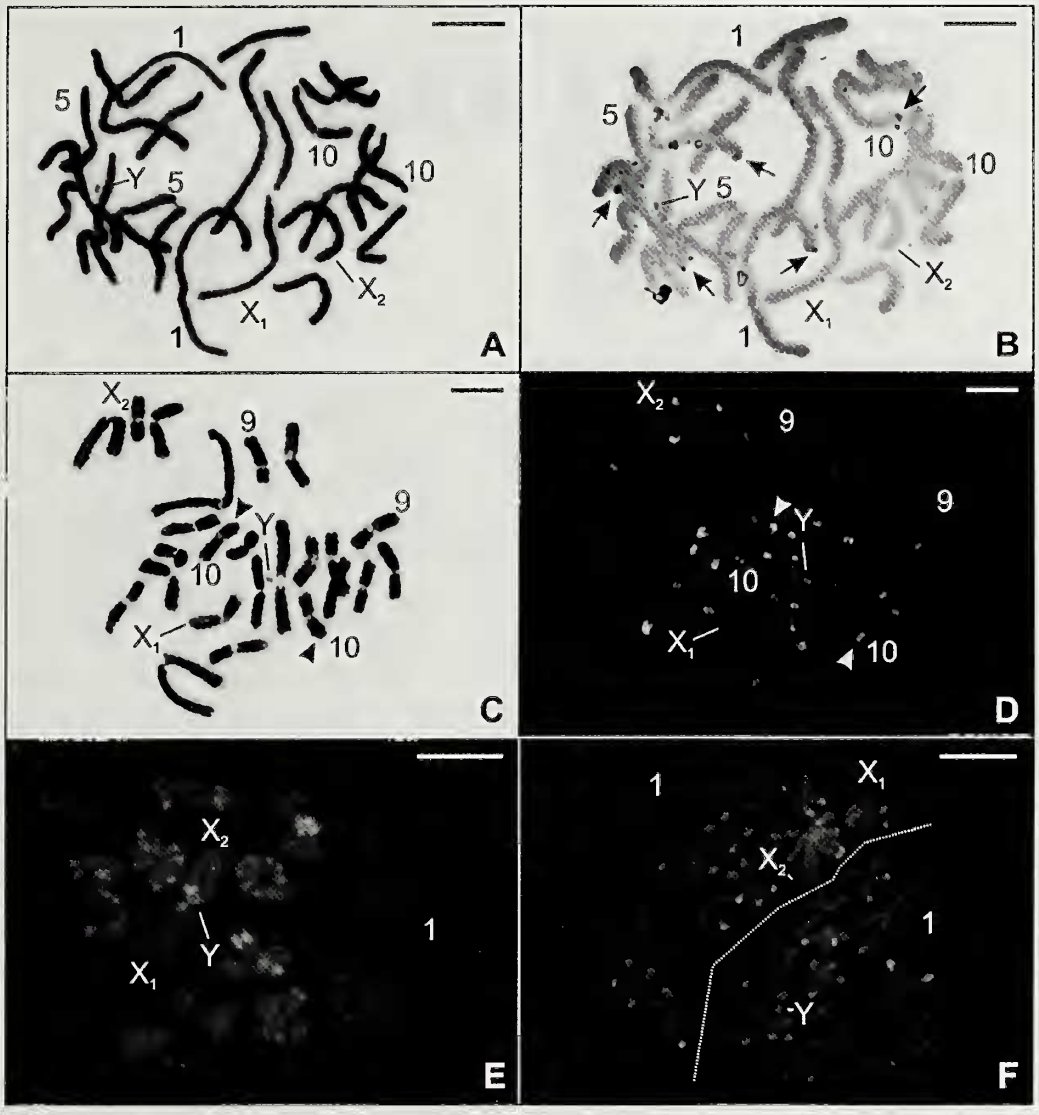

Figure 2.—Testicular cells of *Kukulcania hibernalis*, stained with Giemsa (A, C), silver-impregnated (B) and DAPI C-banded (D–F). A, B. Mitotic metaphase, revealing nucleolar organizer regions (arrows) on the terminal regions of pairs 1, 5 and 10. C, D. Mitotic metaphase, showing the predominance of constitutive heterochromatin in the terminal regions of the chromosomes. The arrowheads in pair 10 point to heterochromatin colocalized with the secondary constrictions. E, F. Diplotene and metaphase II, respectively, exhibiting pericentromeric heterochromatin in X_2 and the totally heterochromatic Y chromosome. Scale = 10 μm.

same cell, with the number of active NORs varying from two to five. Additionally, the NORs located on pair 10 were coincident with secondary constriction observed in Giemsa-stained cells. DAPI C-banded testicular cells showed constitutive heterochromatin in the short arm terminal region of all autosomal pairs and X_1 and X_2 sex chromosomes, with the exception of pairs 5 and 9 (Fig. 2C, D). Furthermore, blocks of constitutive heterochromatin were visualized in the long terminal region of pairs 4, 5, 6, 7, 8, 10, 11 and X_2 sex chromosome. Additional bands were also found in the short arm interstitial region of pair 4. The Y chromosome was entirely heterochromatic. The constitutive heterochromatin present in the short arm of pair 10 was colocalized with the secondary constriction. In addition to the DAPI C-band pattern observed in mitotic cells, the study of meiotic cells allowed us to identify blocks of heterochromatin in the pericentromeric region of the X_2 sex chromosome (Fig. 2E, F). Metaphase II nuclei confirmed that the DAPI C-banded positive regions of pair 1 were tenuous when compared with those of the other chromosomes.

Misionella mendensis.—Male karyotype analysis of *M. mendensis* revealed 2n = 21, X_1X_2Y metacentric chromosomes, with the exception of pair 1 that was submetacentric (Fig. 3A). The autosomal chromosomes gradually decreased in size, but the X_1 and X_2 sex chromosomes were the largest and the Y chromosome, the smallest elements of the karyotype. Secondary constrictions were located in the long arm interstitial region of pairs 1 and 2. The study of meiotic cells confirmed the diploid number and type of sex chromosome system established for this species (Fig. 3B, D). In diplotene nuclei, the autosomal bivalents exhibited one terminal or interstitial chiasma and the sex chromosomes were associated in a trivalent configuration (Fig. 3B), similar to that observed in *K. hibernalis*. Metaphase II cells showed n = 11, X_1X_2 and n = 10,

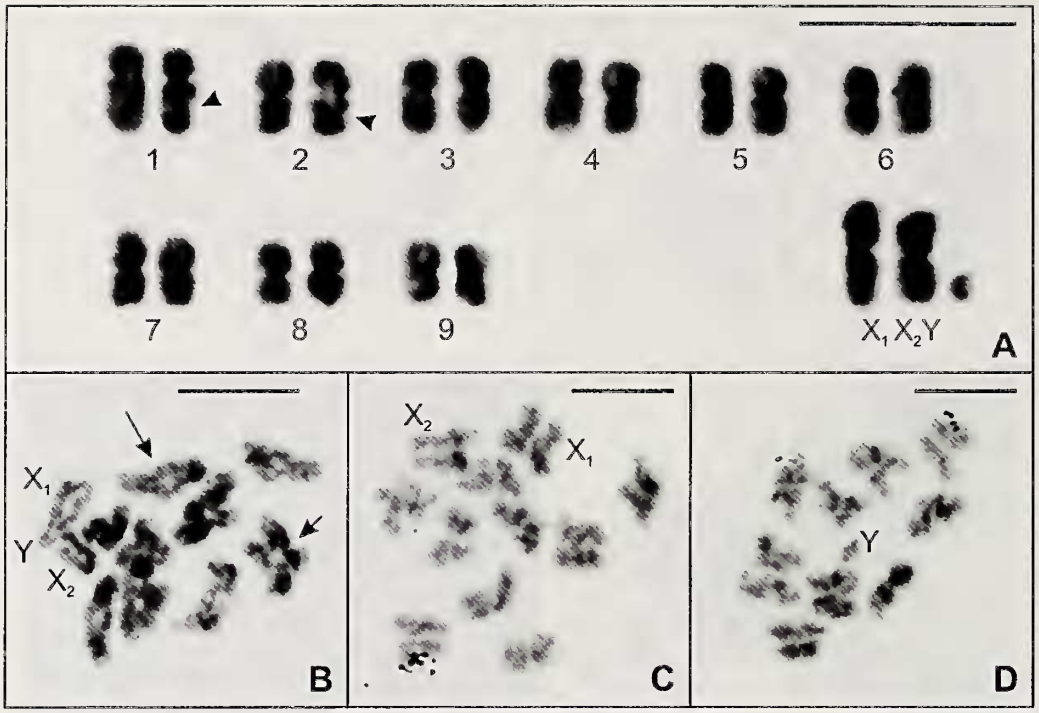

Figure 3.—Testicular cells of *Misionella mendensis*, stained with Giemsa. A. Karyotype, 2n♂ = 21, X_1X_2Y with chromosomes predominantly metacentric. The arrowheads indicate constrictions. B. Diplotene, showing autosomal bivalents with one interstitial (small arrow) or terminal chiasma (large arrow). C, D. Metaphase II with n=11, X_1X_2 and n=10, Y, respectively. Scale = 10 μm.

Y (Fig. 3C, D), indicating the balanced segregation of all chromosomes.

Mitotic cells of embryos examined with Giemsa staining and silver impregnation revealed six NORs on the short arm terminal region of pairs 1, 3 and 5 (Fig. 4A, B). In this species, the constrictions revealed by Giemsa staining were not silver impregnated. Mitotic metaphase cells submitted to C-banding and stained with DAPI showed constitutive heterochromatin in the pericentromeric region of all chromosomes; additional bands were verified in the short arm terminal region of pairs 5 and 8 (Fig. 4C, D). The terminal heterochromatin of pair 5 was located in an NOR-bearing region.

DISCUSSION

The 2n♂ = 21 observed in *M. mendensis* (Prithinae) is the lowest diploid number described until now for Filistatidae. The number of autosomes found in *K. hibernalis* (Filistatinae) is the same as that previously described for one population of this species from Argentina (Rodríguez Gil et al. 2002). The metacentric and submetacentric chromosomal morphology is common for all filistatids, including the species analyzed here, as also occurs for most haplogyne spiders (Araujo et al. 2016).

In Filistatinae, cytogenetic information is available for three species, *Filistata insidiatrix* with 2n♂ = 33, X_1X_2Y (Král et al. 2006), *Kukulcania* aff. *hibernalis* with 2n♂ = 25, X_1X_2Y (Kořínková & Král 2013), and *Kukulcania hibernalis* with 2n♂ = 24, X_1X_20 (Rodríguez Gil et al. 2002) and 2n♂ = 25, X_1X_2Y (present study). In Prithinae, the only species that has been cytogenetically examined is *Misionella mendensis*, 2n♂ = 21, X_1X_2Y. These data show that the highest diploid numbers occur in Filistatinae, but the X_1X_2Y sex chromosome system is a shared characteristic for the species of both subfamilies.

The cytogenetic data did not reveal new information about the Filistatidae affinities, because the X_1X_2Y sex chromosome system found in this family has a morphology and meiotic behavior similar to that described for *Hypochilus* and known for some Synspermiata families (Drymusidae, Pholcidae and Sicariidae) (Král et al. 2006). However, taking into account the presence of the X_1X_2Y system in Hypochilidae and Synspermiata, and considering its absence in Mygalomorphae (Araujo et al. 2016), this system probably reflects the ancestral condition for Araneomorphae (Silva 1988; Oliveira et al. 1996, 1997; Rodriguez Gil et al. 2002; Silva et al. 2002; Král et al. 2006).

The difference between the sex chromosome system described for *K. hibernalis* herein (X_1X_2Y) and from Argentina (X_1X_2) (Rodríguez Gil et al. 2002) could be a case of population variation, however, the possibility of misinterpretation of the sex chromosome system in the paper of Rodríguez Gil et al. (2002) cannot be ruled out. The misidentification of the X_1X_2Y sex chromosome system, due to the tiny size of the Y chromosome, is a problem already pointed out by Král et al (2006). The stage of chromosome condensation in the pictures of *K. hibernalis* presented by Rodríguez Gil et al. (2002) do not allow an unambiguous identification of the Y absence requiring a reanalysis of the Argentinean population.

Cytogenetic data in regard to the identification of specific chromosomal regions are scarce in spiders, considering that less than 10% of the species have been characterized in relation to the distribution of NORs and constitutive heterochromatin (Araujo et al. 2012, 2014, 2015; Forman et al. 2013; Kořínková

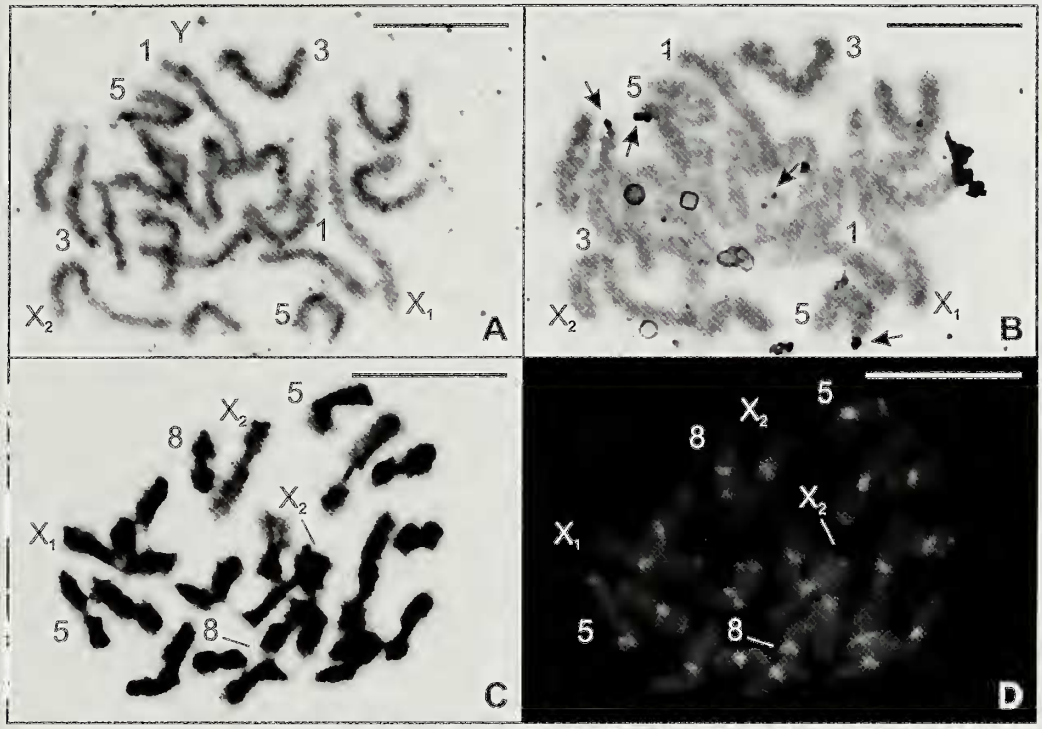

Figure 4.—Embryonic mitotic cells of *Misionella mendensis* stained with Giemsa (A, C), silver-impregnated (B) and DAPI C-banded (D). A, B. Metaphase, $2n♂ = 21$, X_1X_2Y, revealing nucleolar organizer regions (arrow) on the terminal regions of pairs 1, 3 and 5. In B, the Y chromosome is not evident. C, D. Metaphase, $2n♀ = 22$, $X_1X_1X_2X_2$, showing constitutive heterochromatin in the pericentromeric region of all chromosomes. Scale = 10 μm.

& Král 2013; Král et al. 2013). In Mygalomorphae and Araneomorphae, NORs are predominantly located on the terminal region of one to three autosomal pairs. According to Král et al. (2013), this pattern could be a symplesiomorphy of these two suborders of Araneae. Nevertheless, in the Haplogynae lineage, the species possessing X0 and XY systems showed NORs on autosomal chromosomes and the X chromosome, or only on the X chromosome (Král et al. 2006; Oliveira et al. 2007; Araujo et al. 2008). Additionally, the only species with X_1X_2Y sex chromosome system investigated regarding the distribution of NORs, *Hypochilus pococki* Platnick, 1987 (Hypochilidae) with $2n♂ = 29$, showed two pairs of autosomal chromosomes impregnated by silver (Král et al. 2006). Despite the two species of Filistatidae analyzed in this study that showed different diploid numbers, in both species the silver-impregnated regions (Ag-NORs) were located on the terminal region of three autosomal pairs, including pairs 1 and 5. Autosomal NORs are the most common condition in spiders (Araujo et al. 2012)

In spiders, the constitutive heterochromatin commonly exhibits a similar pattern in autosomes and sex chromosomes, occurring in the pericentromeric region (Araujo et al. 2012). In the Filistatidae species studied herein, a clear difference in relation to distribution of constitutive heterochromatin was observed, taking into account that in *K. hibernalis* terminal C-bands were predominant while in *M. mendensis* the positive heterochromatic regions were pericentromeric. This result indicated that in addition to the change in diploid number, the dispersion and/or accumulation of constitutive heterochromatin in specific chromosomal regions may be related to karyotype differentiation in Filistatidae species. Furthermore, a change in the amount of heterochromatin was probably the key event responsible for the heteromorphism observed in the chromosomes of pair 9 of *K. hibernalis*. This is supported by the morphology of the submetacentric element which did not show a positive DAPI C-band in the short arm terminal region. In Haplogynae taxa with a X_1X_2Y sex chromosome system, the Y chromosome is completely heterochromatic. This pattern was observed in *K. hibernalis* and *M. mendensis*, as well in species belonging to other families, e.g., *Loxosceles intermedia* Mello-Leitão, 1934, *Loxosceles laeta* (Nicolet, 1849) (Sicariidae) and *Pholcus phalangioides* (Fuesslin, 1775) (Pholcidae) (Silva et al. 2002; Král et al. 2006).

Although cytogenetic information is restricted to only four species of Filistatidae, the available data revealed that this family has a high karyotype diversity, mainly related to the number of autosomal chromosomes and the distribution of constitutive heterochromatin. Additionally, the diversity in karyotype constitution seems to be a common feature of both basal (Filistatidae) and Synspermiata species, differing from the uniform karyotype pattern observed in most Entelegynae spiders.

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